THE EMBO JOURNAL

One-step generation of T-cell receptor knock-in mice in the TCRβ locus

Jana Bilanovic, Juliana Bortolatto, Susu Duan, Valgerdur Bjørnsdottir, A. Katharina Teetz, Alexandra Thoms, Julian Fishbach, Fabian Schmidt, William Nyberg, Harald Hartweger, Amelia Escolano, Paul Thomas, Gabriel Victora, Angelina Bilate, and Johanne Jacobsen

Corresponding author(s): Angelina Bilate (abilate@rockefeller.edu) , Johanne Jacobsen (j.t.jacobsen@medisin.uio.no)

Review Timeline:

Submission Date:	24th Apr 25
Editorial Decision:	26th Jun 25
Revision Received:	5th Jan 26
Editorial Decision:	13th Feb 26
Revision Received:	10th Mar 26
Accepted:	18th Mar 26

Editor: Ioannis Papaioannou

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Angelina, dear Johanne Tracey,

Thank you again for submitting your manuscript EMBOJ-2025-121127 for consideration by The EMBO Journal, and for your patience during peer review. As I have already informed you, your manuscript has now been seen by three experts in the field, and we have received their detailed and informative reports that I have already shared with you (they are included again below).

I am pleased to say that the referees find the method described in your work significant, relevant, and useful, and mention that the manuscript is detailed and well-written. While they are largely supportive of the work, they also identify a number of limitations and raise a few concerns, providing detailed suggestions for strengthening the work and the manuscript further. We think that the reports are balanced, fair, and constructive, and are glad to hear that you also find them constructive and the points raised largely addressable.

Given the referees' supportive comments and positive recommendations, as well as your feedback, I would like to invite you to submit a revised version of your manuscript taking the referees' suggestions on board, along with a detailed point-by-point response addressing all referees' comments. I should add that it is The EMBO Journal policy to allow only a single round of major revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. As we have already discussed, I kindly encourage you to share with me a brief point-by-point response to the referees' comments already during the initial stages of your revision, commenting particularly on any points you do not agree with or cannot address during the revision, to discuss how to best proceed. Please also feel free to contact me if you have any questions or other comments you would like to discuss with me.

We generally allow three months as standard revision time (September 25, 2025). As a matter of policy, competing manuscripts published during this period will not negatively impact our assessment of the conceptual advance presented by your study. However, we request that you contact us as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we will be able to grant an extension.

Thank you for the opportunity to consider your work for publication in The EMBO Journal. I look forward to your revision.

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor, The EMBO Journal
i.papaioannou@embojournal.org

*****
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*****

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2. Please note that no statistics should be calculated and shown in Figures if n=2. Please also note that each p value should be reported as an exact value.

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Referee #1:

The manuscript by Bilanovic et al. describes a very efficient generation of a knock-in mouse for the α/β T cell receptor using AAV and CRISPR/Cas9 technology in mouse zygotes. The technology described is (surprisingly) the first-of-its-kind and therefore essential to be published. The authors correctly stated that transgenic technology used for many TCRs causes various problems, which are addressed by the "new" technology. The technology, including detailed technical information, is very well described. The results describe all the essential analyses one would like to have to appreciate the physiologic expression of the TCR as well as the functions of the T cells that develop in these mice. An interesting effect on T cell differentiation is also described after influenza infection, which, although not essential for the novelty and importance of the manuscript, is worth noting.

Here are my minor comments in random order:
- As it appears from Figure 3B, just 62% of T cells bind to the tetramer as a clearly distinguishable population. This is in conflict to the notion that allelic exclusion is almost complete, as it is convincingly shown by the Vβ analysis in Fig. 3 F. This needs at least a discussion. Maybe the authors can provide data on gating for tetramer-negative T cells and further analysis for Vbeta chain expression by FACS. It would not be surprising (and not really a problem) that those T cells escaped allelic exclusion and expanded in the periphery. The choices for Vß rearrangement are plenty, as the second allele is intact and actually, the DJCβ1 locus is also intact. A careful reanalysis and/or discussion would resolve a certain degree of incomprehension among experts in thymus development and allelic exclusion.
- Fig. 3 E and F could profit from either reorganisation or clearer labeling. It isn't easy to understand, especially with the short accompanying text in the results section. Values for βKI are difficult to understand, given that only 38% of T cells are tetramer positive. The labeling for the schematic of the signaling apparatus must be g) in the legend
- Also for Fig. 3: Why not showing a TCRα/TCRβ FACS staining (on CD3 pregated cells) for the knock-in chains?
- The reference Brady et al. should be replaced by an earlier publication showing RAG downregulation PMID 8608942

- in the light of the mentioning of the-first-of-its-kind character of the knock-in approach for T cell receptor chains, it should be mentioned that a fully functional δ TCR chain was knocked in at the correct side in the locus and showed correct developmental expression, before PMID 37182210
- The FACS staining of wildtype mouse thymus for CD4 and CD8 is grossly deviating from "normal" percentages in the literature for DN (usually 3-5%) and SP (7-10% for CD4 and 5-8% for CD8. This should be reanalysed.

Referee #2:

The authors clearly outlined the history, utility, and pitfalls of transgenic TCR mice in research. With the advancements of CRISPR/Cas9 technology, it is logical to use this approach to improve on monoclonal TCR generation methods. Rollins, et al made knock-in TCR mice by targeting the abTCR to the Tra locus, which leads to more physiological thymocyte development, but requires endogenous rearrangement of the TCRb. This subsequently results in the concern of dual TCRb expression which could confound results. Here, Bilanovic et al. targeted the Trb locus with CRISPR/Cas9 and AAV6 to knock in abTCR from an NP-specific CD4+ T cell and generated two mouse strains, each utilizing a different promoter. The resulting T cells had accelerated thymocyte development due to premature TCR expression, but the authors showed that there was efficient allelic exclusion of the other Trb locus. This provides an additional tool for researchers to generate monoclonal TCR mice, depending on the needs for the project.
Overall, the authors very clearly outline their methods and show the KI TCR cells differentiate into different T helper subsets in vitro and in vivo. Comparison of the KI TCR CD4+ T cells to a TG CD4+ T cell with the same specificity but different TCR sequence does not seem to be the best comparison, especially when speculating about results being due to TCR affinity when no affinity measurements were taken (lines 265-268). I don't want to propose that the authors need to make a traditional TG mouse with their selected TCR sequence, or that they must make new KI TCR mice with the old TG TCR, but perhaps comparing the ratio of tetramer MFI:TCRb MFI of naïve NP+ T cells from the three groups could add more information about the relative avidities of the TCRs.
The manuscript could be strengthened with more information about the 30% of T cells that do not bind tetramer in the KI TCR mice, and perhaps re-analysis of the T cell subsets in the immunization and influenza infection experiments:
Approximately 70% of the KI TCR CD4+ T cells bind tetramer but the authors do not expound on this result. Is this due to poor allelic exclusion of the KI Trb or Tra locus and rearrangement of the endogenous loci? The TCR the authors chose utilizes TRBV3, which I believe is also known as Vb16. Vb16 was not used in Figure 3 F when characterizing the TCRb usage, I think because there is no commercially available antibody. If so, the authors could explicitly state this to explain why they are relying on tetramer binding to identify cells expressing the KI TCR. If the 30% of CD4+ cells that do not bind tetramer have an endogenously rearranged TCR, the Va2 and TCRb usage should look like the WT CD4+ T cells in Fig 3 E. The authors should show these data. Additionally, if the endogenous locus rearranges in some mice, there should be some CD8+ abT cells as well as gdT cells in these KI mice, which should be absent in a traditional TG T cell mouse. Please show this data as well. Finally, if KI mice are crossed to generate homozygous KI mice, does the percent of NP+ in CD4+ T cells jump to 100% in KI mice?
In some retrogenic approaches where the TCRa and TCRb chains are linked by p2A, the surface level of TCR is lower than in WT mice. Please show surface staining of the TCR, for example with H57.597 of the aKI, bKI, Tg, and WT mice, both for the thymus in Figure 2 and for peripheral tissues in Figure 3.
In vaccination and infection experiments with transferred KI TCR cells, in Fig 4 D, F and G, the data are presented as the percent of Tfh that are CD45.2 (Transferred TG or KI cells). What percent of TG or KI TCR CD4+ T cells become Tfh (rather than showing % of Tfh that are TG/KI)? The proportion of transferred cells in each subset could be influenced by the relative ability of the cells to expand in vivo. Additionally, in Fig 4 H the cells are pre-gated on TG/KI TCR cells and displayed as the % Tbet+ within CXCR5- PD1- gate. There are more Tfh in the KI TCR populations, so plotting % Tbet this way is inflating proportions a bit. It would be more accurate to present these data as % Tbet+ of all TG / TCR KI CD4+.
Minor notes:
• Fig 2 F - the slash in the Y axis label (CD24neg/TCRb+) was confusing - it implies it's a ratio
• The methods section and Fig 1 A state the mice were infected with the X31 strain of IAV, however the results section refers to PR8 (line 117). If it indeed was X31, the body of the text should be corrected.
• Typo on line 250: "the latter were been shown" should be "the latter were shown" or "the latter have been shown"
• Typo line 267: "differences in the affinities of each TCR to for pMHC" remove "to"
• Line 466: embryo is missing "e"

Referee #3:

General remarks:
In the manuscript titled "One-step generation of TCR knock-in mice targeted to the TCRβ locus results in functional mature T Cells", Bilanovic et al. describe the generation of TCR-transgenic mice through AAV-mediated CRISPR editing, inserting the transgene in the endogenous gene locus. The manuscript is well written, and the data are clearly visualized. The study is the second study describing TCR knock-in mice with orthotopic insertion, but in contrast to Rollins et al. the authors target the beta locus to prevent dual TCR beta chain occurrence. The described approach is highly relevant for immunological research in

mouse models. However, the interpretation of biological results is compromised by the lack of suitable control mice expressing the same TCR in a non-physiological setting.

Major points:
It is nice that the authors developed a mouse model with a novel TCR (which first had to be experimentally identified). However, a significant downside of this choice is that the results from the TCR knock-in mice cannot be compared to conventional transgenic mice. Orthotopic knock-in of, e.g., the SMARTA or the OT-II TCR would have enabled actual benchmarking against conventional transgenic TCR regulation (arguably with more constitutive TCR gene expression through extrinsic gene promoters).
Using such model TCRs would also have made it easier to analyze the behavior of TCR-knock-in T cells upon chronic antigen exposure (in chronic viral infection or tumor models), which represent settings in which the unique benefit of physiological TCR regulation might have become particularly apparent (since T cells may be better able to protect themselves from exhaustion through TCR downregulation). This would have enabled the authors to test whether the in-vivo phenotypes described by Eyquem et al., Nature 2017 (https://doi.org/10.1038/nature21405, this study should also be cited) in xenograft models would hold true with a knock-in TCR in a syngeneic setting.
While it would be clearly beyond the scope of the manuscript to generate another knock-in mouse line with a model CD4 TCR, the authors should be able to generate retrogenic mice for the influenza TCR, and thereby use a constitutively active gene promotor according to the conventional protocol described by Holst et al., Nat Protocols 2006 using viral transduction of hematopoietic stem cells (https://doi.org/10.1038/nprot.2006.61; this study should also be cited). The alternative transgenic TCR that is specific for the same influenza epitope that is not introduced until Fig. S2 is not suitable in this regard since it is a different TCR. Retrogenic mice with the same TCR would allow a more comparative baseline assessment of TCR expression levels, CD5 expression levels, etc. that the authors performed in the first figures and of the in-vivo reactivity shown in Fig. 4 (when the authors could only compare the knock-in TCR to polyclonal WT TCRs or the alternative transgenic TCR).
The authors could then also engineer recombinant viruses leading to chronic infection (like LCMV) to present the influenza epitope that their TCR is targeting, and/or use a tumor model generated in a similar manner. In any case, an in-vitro "stress/exhaustion test" should be possible to conduct, with repetitive antigen challenge over several days. This functional comparison of the knock-in mice to TCR transgenics underlying artificial TCR regulation would significantly strengthen the manuscript. While such a revision is admittedly somewhat extensive, it appears feasible given the authors' expertise.

Minor points:
The study by Hahn et al., Cell Rep 2023 (https://doi.org/10.1016/j.celrep.2023.112253), should be cited, which represents - to my knowledge - the first description of generation of a mouse model with orthotopic TCR insertion, albeit in their case for the γδ TCR. Roth et al., Nature 2018 (https://doi.org/10.1038/s41586-018-0326-5), should also be cited as the first study to show orthotopic TCR knock-in in primary T cells (then human), as well as Schober et al., Nat Biomed Eng 2019 (https://doi.org/10.1038/s41551-019-0409-0), because this study demonstrated the importance of dual chain editing in the setting of TCR knock-in.
In the abstract and at the end of the introduction, it should be mentioned that this study used a CD4 TCR for knock-in to aid the reader.
Fig. 1F: It would also aid the reader if, in the text accompanying this sub-figure, it would be explained that a certain leakiness of endogenous TCR expression is expected, and the degree of leakiness should be interpreted in the context of the unique setting of this knock-in model (comparing the results, for example, to conventional TCR-transgenic mice with or without a RAG1/2 KO background).
Fig. 2H: Please provide exemplary primary data for Fig. 2H (CD5 levels), best in the form of vertically aligned histograms, so that the CD5 expression levels of the monoclonal TCR can be compared to the expression levels of the polyclonal WT population (like later in Fig. 3). For both TCR expression levels and CD5, it would also be nice to see the coefficient of variation of transgenic TCRs vs. polyclonal WT TCRs (the same applies to Fig. 3H-L). For analyses like these, it would also be informative to show (in a supplementary figure) the NP tetramer-negative population of knock-in mice as an internal negative control.
Fig. 3B: Please comment on the potential nature of the CD44- CD62L- population in Q3, which appears even more distinct in the knock-in mice than in WT mice.
Fig. 3D-F: First, please remind the reader about the TRAV/TRBV configuration of the knock-in TCR (TRAV6-7/DV9/TRAJ57 for TCRa chain and TRBV3/TRBD2/TRBJ7 for TCRb chain according to the genetic IMGT nomenclature) and explain which alpha or beta chains this corresponds to in the Wilson nomenclature, which is usually used for v chain-targeting flow cytometry antibodies. This should be v beta 16 for the beta locus according to IMGT (https://www.imgt.org/IMGTrepertoire/index.php?section=LocusGenes&repertoire=nomenclatures&species=mouse&group=TRBV), which is not covered by the shown antibody stainings. This important information is so-far missing. Second, it took me a while to see that in Fig. 3F the y-axis has a different scale, which should be highlighted or differently visualized to make the important point that leaky polyclonal TRAV/TRBV expression is much lower in the knock-in mice than in WT mice. The y-axis should also be changed to "Vα/Vβ(x) expression..." to be more accurate and make the x-axis better comprehendible. The color legend on top is not horizontally aligned. Third, paired alpha-beta TCR repertoire sequencing would add significantly to this part of the manuscript, since it would not only validate correct expression of the desired TCR in knock-in mice, but also enable a more comprehensive assessment of the polyclonal background expression of other TCRs genes. This aspect appears particularly important given that the engineering aspect of the manuscript at hand represents an advance beyond the study of Rollins et al., Nat Comm 2023, mostly because the endogenous beta chain is targeted.
The helpful brief speculation of the authors why the v beta promotor knock-in mice did not show leakiness for endogenous beta

chains should appear earlier in the text (when Fig. 3F is introduced) since otherwise the reader is left wondering why this could be the case until the end of the text's paragraph is reached.
The figure legend of Fig. 3 is wrongly annotated from Fig. 3G onwards.
Figure 4: As outlined in the major comments above, these analyses would considerably benefit from a retrogenic control of the same TCR that was used for the knock-in TCR. With the present data at hand, it is hard to interpret the differences to the alternative transgenic TCR since TCR avidity differences may mask regulation-effects, as openly discussed by the authors themselves. In-vitro testing of peptide sensitivity would also be an interesting readout in this context (both to see differences in TCR regulation when the same TCR is used through, e.g., knock-in and retrogenic mice, and to see differences between the knock-in TCR and the alternative TCR to validate the assumed avidity differences - it is not clear to me from which source the authors take their confidence in the differential avidity given that the TCR used for the knock-in mice was newly identified in the present study).
In the discussion, the role of TCR affinity remains unclear given the limitations of same-TCR comparisons (as outlined above). The insinuated superiority of the presented workflow to the approach by Rollins et al. - although it is intuitive - should also be phrased more carefully because experimental evidence for less heterogeneity and less confounding affects with the system of the manuscript at hand could not be sufficiently shown. The discussion should also include a paragraph that addresses the question how physiological the approach with inserted v alpha or v beta promotors is compared to natural gene regulation. In this regard, it should be an interesting food for thought for the reader that in natural polyclonal populations each TCR that has a different V segment underlies differential gene regulation.

We thank all the reviewers for their thorough evaluation of our manuscript and helpful suggestions. We have addressed the concerns raised by the reviewers in the revised version, and we believe the manuscript is much improved. All text changes in the new version of the manuscript are now marked in blue. We have also edited the text and figures to clarify our main message which is the development of a streamlined approach to generate TCR monoclonal knock-in mice. Please see our point-by-point responses below.

Referee #1:

The manuscript by Bilanovic et al. describes a very efficient generation of a knock-in mouse for the α/ß T cell receptor using AAV and CRISPR/Cas9 technology in mouse zygotes. The technology described is (surprisingly) the first-of-its-kind and therefore essential to be published. The authors correctly stated that transgenic technology used for many TCRs causes various problems, which are addressed by the "new" technology. The technology, including detailed technical information, is very well described. The results describe all the essential analyses one would like to have to appreciate the physiologic expression of the TCR as well as the functions of the T cells that develop in these mice. An interesting effect on T cell differentiation is also described after influenza infection, which, although not essential for the novelty and importance of the manuscript, is worth noting.

We thank the reviewer for this positive evaluation and addressed all the minor comments raised by the reviewer below.

Here are my minor comments in random order:

- As it appears from Figure 3B, just 62% of T cells bind to the tetramer as a clearly distinguishable population. This is in conflict to the notion that allelic exclusion is almost complete, as it is convincingly shown by the Vβ analysis in Fig. 3 F. This needs at least a discussion. Maybe the authors can provide data on gating for tetramer-negative T cells and further analysis for Vbeta chain expression by FACS. It would not be surprising (and not really a problem) that those T cells escaped allelic exclusion and expanded in the periphery. The choices for Vß rearrangement are plenty, as the second allele is intact and actually, the DJCβ1 locus is also intact. A careful reanalysis and/or discussion would resolve a certain degree of incomprehension among experts in thymus development and allelic exclusion.

We agree with the reviewer that our description of escape was poorly phrased. We have modified the text to indicate that allelic exclusion is indeed not 100%, adding new text to the results and discussion that clarifies our interpretation of the data in the new Fig. 3F.

As the reviewer points out, because of how the pre-rearranged TCRαβ was inserted into the *Trb* locus, escapees can still rearrange the other allele. Nevertheless, roughly 60-70% of $CD4^+$ T cells in the TCR KI mice bind to the NP tetramer. To assess the nature of these “escapees” (non-binders) we analyzed TCRα/β expression by FACS of blood T cells and by single-cell TCR sequencing (scTCRseq) of splenic T cells. The new Fig. 3E-F show the protein expression of non-KI TCRβs and of TCR Vα2 among NP-tetramer binders and non-binders. Among binders, the expression of non-KI Vs was close to zero. In contrast, a larger fraction of the non-tetramer binders expressed non-KI TCRs, indicating that allelic exclusion occurs to some extent, but it is not 100%. We have edited the text accordingly.

To complement this analysis, we performed scTCRseq of NP-tetramer binders and non-binders. Our data show that, whereas NP binders carry the correct TCR KI rearrangement and are thus monospecific, roughly half of non-binders carry other, unique non-KI CDR3 rearrangements (see new Fig. 3G), indicating that rearrangement and expression of non-KI alleles can lead to loss of tetramer binding, possibly by mispairing between KI and non-KI TCR chains.

- Fig. 3 E and F could profit from either reorganisation or clearer labeling. It isn't easy to understand, especially with the short accompanying text in the results section. Values for βKI are difficult to understand, given that only 38% of T cells are tetramer positive. The labeling for the schematic of the signaling apparatus must be g) in the legend

Thank you for this suggestion and we apologize for the confusion. We modified the figure to clearly indicate the antibodies used and discern Vα2 from all other Vβ stainings. Additionally, we have also included the Vβ staining for both binders and non-binders (see new Fig. 3F). We have also removed the schematic for TCR signaling and expanded the results section to aid the reader.

- Also for Fig. 3: Why not showing a TCRα/TCRβ FACS staining (on CD3 pregated cells) for the knock-in chains?

We unfortunately are unable to stain specifically for the TCRα/β of our knock-in because monoclonals are not available for these particular V-segments. This is why we need to use tetramer staining along with the “negative” staining for the Vα2 and pan-Vβ, which identifies escapees. We added an explanatory note to this end to the text describing Fig. 3.

- The reference Brady et al. should be replaced by an earlier publication showing RAG downregulation PMID 8608942

We thank the reviewer for providing the PMID and have replaced the citation.

- in the light of the mentioning of the-first-of-its-kind character of the knock-in approach for T cell receptor chains, it should be mentioned that a fully functional δ TCR chain was knocked in at the correct side in the locus and showed correct developmental expression, before PMID 37182210

We thank the reviewer for this comment and have added this to the introduction.

- The FACS staining of wildtype mouse thymus for CD4 and CD8 is grossly deviating from "normal" percentages in the literature for DN (usually 3-5%) and SP (7-10% for CD4 and 5-8% for CD8. This should be reanalysed.

Thank you for pointing this out. We have included more mice in this analysis and reanalyzed the data. We added a new representative FACS plot in Fig 2B to reflect this change.

Referee #2:

The authors clearly outlined the history, utility, and pitfalls of transgenic TCR mice in research. With the advancements of CRISPR/Cas9 technology, it is logical to use this approach to improve on monoclonal TCR generation methods. Rollins, et al made knock-in TCR mice by targeting the abTCR to the Tra locus, which leads to more physiological thymocyte development, but requires endogenous rearrangement of the TCRb. This subsequently results in the concern of dual TCRb expression which could confound results. Here, Bilanovic et al. targeted the Trb locus with CRISPR/Cas9 and AAV6 to knock in abTCR from an NP-specific CD4+ T cell and generated two mouse strains, each utilizing a different promoter. The resulting T cells had accelerated thymocyte development due to premature TCR expression, but the authors showed that there was efficient allelic exclusion of the other Trb locus. This provides an additional tool for researchers to generate monoclonal TCR mice, depending on the needs for the project.

We thank the reviewer for this positive evaluation and addressed all the comments raised by the reviewer below.

- Overall, the authors very clearly outline their methods and show the KI TCR cells differentiate into different T helper subsets in vitro and in vivo. Comparison of the KI TCR CD4+ T cells to a TG CD4+ T cell with the same specificity but different TCR sequence does not seem to be the best comparison, especially when speculating about results being due to TCR affinity when no affinity measurements were taken (lines 265-268). I don't want to propose that the authors need to make a traditional TG mouse with their selected TCR sequence, or that they must make new KI TCR mice with the old TG TCR, but perhaps comparing the ratio of tetramer MFI:TCRb MFI of naïve NP+ T cells from the three groups could add more information about the relative avidities of the TCRs.

The reviewer is correct to point out that we cannot directly compare the properties of two TCRs with different CDR3 rearrangements. Our goal in including the TCR transgenic was to broadly benchmark our new method against a more traditional approach, not to compare specifics, but we agree that our language did not make this clear. We have modified the text and figures to remove these direct comparisons between models. The new edits are indicated in blue in the text (please see new text edits regarding Fig. 4).

The manuscript could be strengthened with more information about the 30% of T cells that do not bind tetramer in the KI TCR mice, and perhaps re-analysis of the T cell subsets in the immunization and influenza infection experiments:

- Approximately 70% of the KI TCR CD4+ T cells bind tetramer but the authors do not expound on this result. Is this due to poor allelic exclusion of the KI Trb or Tra locus and rearrangement of the endogenous loci?

We agree with the reviewer that our description of escape was insufficient. As the reviewer points out, about 70% of CD4 T cells in the TCR KI mice bind to NP tetramer. To more thoroughly assess the nature of the non-KI “escapees” we analyzed TCRα/β expression by FACS of blood T cells and by single-cell TCR sequencing (scTCRseq) of splenic T cells. The new Fig. 3E-F shows the protein expression of non-KI TCRβs and of TCR Vα2 among NP-tetramer binders and non-binders. Among binders, the expression of non-KI Vs was close to zero. In contrast, a larger fraction of the non-tetramer binders expressed non-KI TCRs, indicating that allelic exclusion occurs to some extent, but it is not 100%.

To complement the FACS analysis, we performed scTCRseq of NP-tetramer binders and non-binders. Our data show that, whereas NP binders carry the correct TCR-KI rearrangement and are thus monospecific, roughly half of non-binders carry other, unique non-KI CDR3 rearrangements (see new Fig. 3G-H), indicating that rearrangement and expression of non-KI alleles can lead to loss of tetramer binding, possibly by mispairing between KI and non-KI TCR chains.

Additionally, we now show in the new Fig. 4C and G that donor-derived Tfh cells recovered after immunization or infection are all NP-tetramer binders.

- The TCR the authors chose utilizes TRBV3, which I believe is also known as Vb16. Vb16 was not used in Figure 3 F when characterizing the TCRb usage, I think because there is no commercially available antibody. If so, the authors could explicitly state this to explain why they are relying on tetramer binding to identify cells expressing the KI TCR. If the 30% of CD4+ cells that do not bind tetramer have an endogenously rearranged TCR, the Va2 and TCRb usage should look like the WT CD4+ T cells in Fig 3 E. The authors should show these data. Additionally, if the endogenous locus rearranges in some mice, there should be some CD8+ abT cells as well as gdT cells in these KI mice, which should be absent in a traditional TG T cell mouse. Please show this data as well. Finally, if KI mice are crossed to generate homozygous KI mice, does the percent of NP+ in CD4+ T cells jump to 100% in KI mice?

The text has been updated to clarify this point. Indeed, no commercially available antibody exists to stain our specific Vβ16 chain. We hope the new layout of figure 3F also clarifies this issue. We have also added new flow cytometry data for γδ and $CD8^+$ T cells proportions in TCR KI mice (see new Supplemental Fig. 2).

While we see about 70% of NP-tetramer binders in heterozygous mice one would expect that homozygous mice would have less escapees. We have not intercrossed the TCR KI mice to homozygosity, but we predict that this would indeed increase the proportion of NP-binders, as reported previously for classic transgenic models (PMID: 7520367).

- In some retrogenic approaches where the TCRa and TCRb chains are linked by p2A, the surface level of TCR is lower than in WT mice. Please show surface staining of the TCR, for example with H57.597 of the aKI, bKI, Tg, and WT mice, both for the thymus in Figure 2 and for peripheral tissues in Figure 3.

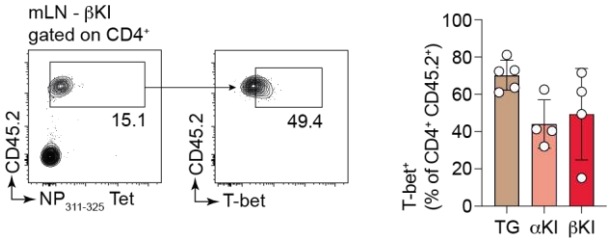
We now show this data in Figure 2G and 3K, and, for clarification, we have added overlaid histograms showing TCRβ expression in WT and TCR KI mice.

- In vaccination and infection experiments with transferred KI TCR cells, in Fig 4 D, F and G, the data are presented as the percent of Tfh that are CD45.2 (Transferred TG or KI cells). What percent of TG or KI TCR CD4+ T cells become Tfh (rather than showing % of Tfh that are TG/KI)? The proportion of transferred cells in each subset could be influenced by the relative ability of the cells to expand in vivo.

We have now also added the flow cytometry plots and bar graphs showing the frequency of Tfh out of all transferred cells to new Figure 4C and 4G.

- Additionally, in Fig 4 H the cells are pre-gated on TG/KI TCR cells and displayed as the % Tbet+ within CXCR5- PD1- gate. There are more Tfh in the KI TCR populations, so plotting % Tbet this way is inflating proportions a bit. It would be more accurate to present these data as % Tbet+ of all TG / TCR KI CD4+.

It was not our intention to inflate the proportion of Tbet-expressing cells but rather highlight the ability of the transferred cells to not only differentiate into Tfh but also into Th1 cells. We would like to clarify that we aimed to characterize the phenotypic identity of the non-Tfh compartment rather than the absolute yield of Th1 cells. Both TG and KI T cells can differentiate into Th1 cells in the non-Tfh compartment. Our gating strategy allowed us to characterize the fate of the transferred cells that did not differentiated into Tfh. Please see the **Reviewer Figure 1** below showing the frequency of Tbet-expressing cells among all transferred cells.



Reviewer Figure 1. Expression of T-bet among transferred TCR TG or TCR KI T cells. Representative flow cytometry plots showing T-bet expression on transferred $CD4^+$ NP^+ $CD45.2^+$ T cells. The plots are displaying the same representative sample as shown in Fig. 4H, now applying the T-bet gate on all transferred cells. Data are pooled from two independent experiments. Data and error bars represent the mean ± s.e.m.

Minor notes:

• Fig 2 F - the slash in the Y axis label (CD24neg/TCRb+) was confusing - it implies it's a ratio
• The methods section and Fig 1 A state the mice were infected with the X31 strain of IAV,

however the results section refers to PR8 (line 117). If it indeed was X31, the body of the text should be corrected.

• Typo on line 250: "the latter were been shown" should be "the latter were shown" or "the latter have been shown"

• Typo line 267: "differences in the affinities of each TCR to for pMHC" remove "to"

• Line 466: embryo is missing "e"

Thank you for noticing our mistakes. All of them have now been corrected.

Referee #3:

General remarks:

In the manuscript titled "One-step generation of TCR knock-in mice targeted to the TCRβ locus results in functional mature T Cells", Bilanovic et al. describe the generation of TCR-transgenic mice through AAV-mediated CRISPR editing, inserting the transgene in the endogenous gene locus. The manuscript is well written, and the data are clearly visualized. The study is the second study describing TCR knock-in mice with orthotopic insertion, but in contrast to Rollins et al. the authors target the beta locus to prevent dual TCR beta chain occurrence. The described approach is highly relevant for immunological research in mouse models. However, the interpretation of biological results is compromised by the lack of suitable control mice expressing the same TCR in a non-physiological setting.

We thank the reviewer for their positive comments and thorough reading of our manuscript. We believe the new data and text clarifies how we validated our approach to generate TCR KI mice.

Major points:

It is nice that the authors developed a mouse model with a novel TCR (which first had to be experimentally identified). However, a significant downside of this choice is that the results from the TCR knock-in mice cannot be compared to conventional transgenic mice. Orthotopic knock-in of, e.g., the SMARTA or the OT-II TCR would have enabled actual benchmarking against conventional transgenic TCR regulation (arguably with more constitutive TCR gene expression through extrinsic gene promoters).

Using such model TCRs would also have made it easier to analyze the behavior of TCR-knock-in T cells upon chronic antigen exposure (in chronic viral infection or tumor models), which represent settings in which the unique benefit of physiological TCR regulation might have become particularly apparent (since T cells may be better able to protect themselves from exhaustion through TCR downregulation). This would have enabled the authors to test whether the in-vivo phenotypes described by Eyquem et al., Nature 2017 (https://doi.org/10.1038/nature21405, this study should also be cited) in xenograft models would hold true with a knock-in TCR in a syngeneic setting.
While it would be clearly beyond the scope of the manuscript to generate another knock-in mouse line with a model CD4 TCR, the authors should be able to generate retrogenic mice for the influenza TCR, and thereby use a constitutively active gene promotor according to the conventional protocol described by Holst et al., Nat Protocols 2006 using viral transduction of hematopoietic stem cells (https://doi.org/10.1038/nprot.2006.61; this study should also be cited). The alternative transgenic TCR that is specific for the same influenza epitope that is not introduced until Fig. S2 is not suitable in this regard since it is a different TCR. Retrogenic mice with the same TCR would allow a more comparative baseline assessment of TCR expression

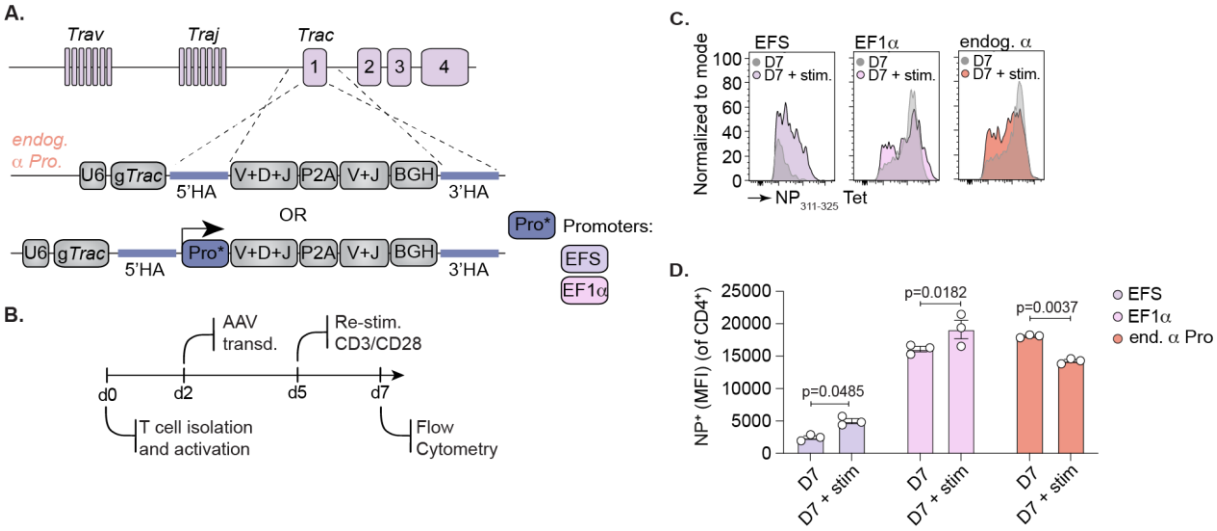
levels, CD5 expression levels, etc. that the authors performed in the first figures and of the in-vivo reactivity shown in Fig. 4 (when the authors could only compare the knock-in TCR to polyclonal WT TCRs or the alternative transgenic TCR). The authors could then also engineer recombinant viruses leading to chronic infection (like LCMV) to present the influenza epitope that their TCR is targeting, and/or use a tumor model generated in a similar manner. In any case, an in-vitro "stress/exhaustion test" should be possible to conduct, with repetitive antigen challenge over several days. This functional comparison of the knock-in mice to TCR transgenics underlying artificial TCR regulation would significantly strengthen the manuscript. While such a revision is admittedly somewhat extensive, it appears feasible given the authors' expertise.

We agree with the reviewer that we cannot directly compare the current TG mice with our KI mice as they carry different CDR3 rearrangements, and we have removed the passages that made such comparisons in the revised version. Our intention with the TG mice was to provide a broad benchmark for our novel method in comparison with traditional approaches. Thus, we opted to generate mice using a TCR newly identified from the BALF of infected mice, which would be more useful to the field than making a second version of the same TCR already available. We have edited the text accordingly to clarify these points.

The primary goal of our effort was to create and validate an approach that uses CRISPR/Cas9 and AAVs to insert TCRs into an endogenous locus. The major advantage of these mice is that they are genetically defined, use endogenous regulatory elements, and are easier to generate than traditional TCR transgenics. While comparisons of the biology of T cells obtained with each approach would be interesting in principle, we would argue that any differences revealed by such comparisons would likely reflect problems with random and variable transgene integration in the transgenic or retrogenic, rather than with our targeted approach. For example, retrogenic mice can display lower TCR expression levels than traditional transgenic mice (PMID: 22348644) introducing other variables (TCR surface density) that would make it difficult to discern the effect of the expression system itself. We therefore believe that additional experiments with retrogenics (which differ from germline modified T cells in countless ways in addition to promoters and integration loci) to be beyond the scope of the present study.

Nevertheless, to investigate the question of endogenous vs. exogenous promoters, we expressed the same TCRα/β rearrangement used in our KI mice under the control of endogenous or strong exogenous promoters in mouse primary T cells using the Ark313 synthetic adeno-associated virus (PMID: 36638795). This method is a direct derivative of the one used by Eyquem et al. 2017 (PMID: 28225754) to target and express a CAR from the *Trac* locus. Thus, the TCRα promoter is the true endogenous control promoter in this case. In addition to the endogenous TCRα promoter, we chose a strong constitutive promoter commonly used for transgene expression, EF1α (PMID: 2210382), and the truncated weaker EFS promoter (PMID: 2210382). The latter is still considered a constitutive (housekeeping gene) promoter and is used for transgene expression (PMID: 39437851). This strategy allowed us to compare the impact of different promoters driving TCR expression in response to TCR engagement and restimulation *in vitro*. The experimental design is detailed in PMID: 36638795 and in the **Reviewer Figure 2** below. In short, primary T cells were isolated from Cas9-mice, activated with anti-CD3/CD28, and transduced with AAV donor vectors designed to insert our NP-specific TCRα/β cassette into the *Trac* locus. The TCR cassette was driven by one of the three different promoters mentioned above. Expression of TCR was then assessed after one or two rounds of TCR stimulation (see **Reviewer Figure 2**). Splenic TCR KI T cells expressing TCR under endogenous *Trac* promoter displayed downregulation of TCR upon anti-CD3/CD28 stimulation, which was not observed using a strong constitutive promoter. These results suggest that strong promoters may lock the T cells in an “on position,” maintaining TCR expression upon restimulation, while under the endogenous *Trac* locus control, TCR expression is physiologically downregulated upon TCR restimulation (see **Reviewer Figure 2**). Since this is a very different system and not directly related to the *Trb* targeting approach

we used to generate germline TCR KI mice we opted for not showing this data in the manuscript. Nevertheless, these results highlight the utility of driving TCR expression through endogenous promoters.



Reviewer Figure 2. Effect of promoter choice on TCR expression upon restimulation. A) Schematic representation of AAV template designs for *Trac* targeting. A template for NP-TCR expression from *Trac* was designed by using homology arms targeting exon 1 of *Trac* and a U6 promoter expressing a sgRNA targeting *Trac.* TCRβ and TCRα chains were separated by a P2A cleavable linker. Additional templates were designed to substitute *Trac*-regulation of the NP-TCR with inserted promoters, the NP-TCR sequences were followed by a stop codon and a BGH poly-A sequence. Two promoters were used for comparisons, EF1α and EFS. All templates were packaged into the Ark313 AAV serotype for T cell transductions. **B**) Splenic T cells were isolated from H11-Cas9 mice and activated using anti-CD3/CD28 dynabeads, followed by AAV transduction 48 hours later. Knock-in frequency was determined at D4 post-T cell activation. Half of the T cells were re-stimulated with an anti-CD3/CD28 dynabeads on D5 post initial T cell activation. Final flow cytometry analysis was performed on D7 post T cell isolation and activation. **C**) Representative histograms showing tetramer expression in CD4⁺ T cells at D7 with or without re-stimulation. **D**) Graph shows the quantification of the MFI for tetramer on CD4⁺ T cells D7 post T cell isolation and/or activation determined by flow cytometry. Tetramer expression was compared with- (D7 + stim.) or without re-stimulation (D7). Results are the mean ± SEM from three different cultures. Significance was assessed using Student's t test.

Minor points:

- The study by Hahn et al., Cell Rep 2023 (https://doi.org/10.1016/j.celrep.2023.112253), should be cited, which represents - to my knowledge - the first description of generation of a mouse model with orthotopic TCR insertion, albeit in their case for the γδ TCR. Roth et al., Nature 2018 (https://doi.org/10.1038/s41586-018-0326-5), should also be cited as the first study to show orthotopic TCR knock-in in primary T cells (then human), as well as Schober et al., Nat Biomed Eng 2019 (https://doi.org/10.1038/s41551-019-0409-0), because this study demonstrated the importance of dual chain editing in the setting of TCR knock-in.

These are relevant references that have now been added in context to the manuscript.

- In the abstract and at the end of the introduction, it should be mentioned that this study used a CD4 TCR for knock-in to aid the reader.

We have specified in the introduction that a CD4⁺ T cell-derived TCR was used to generate the TCR KI mice.

- Fig. 1F: It would also aid the reader if, in the text accompanying this sub-figure, it would be explained that a certain leakiness of endogenous TCR expression is expected, and the degree of leakiness should be interpreted in the context of the unique setting of this knock-in model (comparing the results, for example, to conventional TCR-transgenic mice with or without a RAG1/2 KO background).

Thank you for this suggestion. In the revised manuscript, we added new text in the results and discussion sections to explain that endogenous TCR expression can occur in the TCR KI mice. In addition, to more thoroughly assess the nature of the non-KI "escapees" we analyzed TCRα/β expression by FACS of blood T cells and by single-cell TCR sequencing (scTCRseq) of splenic T cells. The new Fig. 3E-F shows the protein expression of non-KI TCRβs and of TCR Vα2 among NP-tetramer binders and non-binders. Among binders, the expression of non-KI Vs was close to zero. In contrast, a larger fraction of the non-tetramer binders expressed non-KI TCRs, indicating that allelic exclusion occurs to some extent, but it is not 100%.

To complement the FACS analysis, we performed scTCRseq of NP-tetramer binders and non-binders. Our new data showed that, whereas NP binders carry the correct TCR-KI rearrangement and are thus monospecific, roughly half of non-binders carry other, unique non-KI CDR3 rearrangements (see new Fig. 3G-H), indicating that rearrangement and expression of non-KI alleles can lead to loss of tetramer binding, possibly by mispairing between KI and non-KI TCR chains.

- Fig. 2H: Please provide exemplary primary data for Fig. 2H (CD5 levels), best in the form of vertically aligned histograms, so that the CD5 expression levels of the monoclonal TCR can be compared to the expression levels of the polyclonal WT population (like later in Fig. 3). For both TCR expression levels and CD5, it would also be nice to see the coefficient of variation of transgenic TCRs vs. polyclonal WT TCRs (the same applies to Fig. 3H-L). For analyses like these, it would also be informative to show (in a supplementary figure) the NP tetramer-negative population of knock-in mice as an internal negative control.

We have added these data as requested: Figure 2H now includes histograms of CD5 expression in WT and TCR KI T cells. New Supplemental Figure 1 shows the expression of TCRβ and CD5 among NP-tetramer$^-$ T cells from TCR KI mice.

- Fig. 3B: Please comment on the potential nature of the CD44- CD62L- population in Q3, which appears even more distinct in the knock-in mice than in WT mice.

We speculate that this population which constitutes ~7-10% in WT and TCR KI mice may represent cells that have recently been activated or are undergoing homeostatic proliferation, leading to the transient downregulation of CD62L without full upregulation of CD44.

- Fig. 3D-F: First, please remind the reader about the TRAV/TRBV configuration of the knock-in TCR (TRAV6-7/DV9/TRAJ57 for TCRa chain and TRBV3/TRBD2/TRBJ7 for TCRb chain according to the genetic IMGT nomenclature) and explain which alpha or beta chains this corresponds to in the Wilson nomenclature, which is usually used for v chain-targeting flow cytometry antibodies. This should be v beta 16 for the beta locus according to IMGT (https://www.imgt.org/IMGTrepertoire/index.php?section=LocusGenes&repertoire=nomenclatures&species=mouse&group=TRBV), which is not covered by the shown antibody stainings. This important information is so-far missing. Second, it took me a while to see that in Fig. 3F the y-axis has a different scale, which should be highlighted or differently visualized to make the important point that leaky polyclonal TRAV/TRBV expression is much lower in the knock-in mice than in WT mice. The y-axis should also be changed to "Vα/Vβ(x) expression..." to be more accurate and make the x-axis better comprehendible. The color legend on top is not horizontally aligned. Third, paired alpha-beta TCR repertoire sequencing would add significantly to this part of the manuscript, since it would not only validate correct expression

of the desired TCR in knock-in mice, but also enable a more comprehensive assessment of the polyclonal background expression of other TCRs genes. This aspect appears particularly important given that the engineering aspect of the manuscript at hand represents an advance beyond the study of Rollins et al., Nat Comm 2023, mostly because the endogenous beta chain is targeted.

Thank you for raising these important points. We modified the text describing Figure 3 to clearly indicate the antibodies used and discern Vα2 from all other Vβ stainings. Additionally, we have also included the Vβ staining for both NP-tetramer binders and non-binders (see new Fig. 3F). We unfortunately are unable to stain specifically for the TCRα/β of our knock-in because monoclonals are not available for these particular V-segments. This is why we used tetramer staining along with the "negative" staining for the Vα2 and pan-Vβ, which identifies escapees. We added an explanatory note to this end to the text describing Fig. 3.

As for the suggestion of TCR repertoire sequencing, please see reply above for your comment about Fig 1F.

- The helpful brief speculation of the authors why the v beta promotor knock-in mice did not show leakiness for endogenous beta chains should appear earlier in the text (when Fig. 3F is introduced) since otherwise the reader is left wondering why this could be the case until the end of the text's paragraph is reached.

We have moved the explanation and our approach to evaluate the expression of endogenous chains to the beginning of the results section "Physiological peripheral development and efficient TCR allelic exclusion for KI T cells" before we introduce the data shown in Figure 3.

- The figure legend of Fig. 3 is wrongly annotated from Fig. 3G onwards.

We apologize for this oversight; the figure legend has now been corrected.

- Figure 4: As outlined in the major comments above, these analyses would considerably benefit from a retrogenic control of the same TCR that was used for the knock-in TCR. With the present data at hand, it is hard to interpret the differences to the alternative transgenic TCR since TCR avidity differences may mask regulation-effects, as openly discussed by the authors themselves. In-vitro testing of peptide sensitivity would also be an interesting readout in this context (both to see differences in TCR regulation when the same TCR is used through, e.g., knock-in and retrogenic mice, and to see differences between the knock-in TCR and the alternative TCR to validate the assumed avidity differences - it is not clear to me from which source the authors take their confidence in the differential avidity given that the TCR used for the knock-in mice was newly identified in the present study).

Please see our response to the reviewer's first point, above. We agree that we cannot directly compare the biology of our KI T cells to those of transgenic mice using a different TCR and have removed such comparisons from the revised manuscript. As we argue above, we believe comparing our T cells to retrogenics (which differ from germline modified T cells in countless ways in addition to promoters and integration loci) would be beyond the scope of the present manuscript.

- In the discussion, the role of TCR affinity remains unclear given the limitations of same-TCR comparisons (as outlined above). The insinuated superiority of the presented workflow to the approach by Rollins et al. - although it is intuitive - should also be phrased more carefully because experimental evidence for less heterogeneity and less confounding affects with the system of the manuscript at hand could not be sufficiently shown. The discussion should also include a paragraph that addresses the question how physiological the approach with inserted v alpha or v beta promotors is compared to natural gene regulation. In this regard, it should be

an interesting food for thought for the reader that in natural polyclonal populations each TCR that has a different V segment underlies differential gene regulation.

We agree with the reviewer and apologize for the confusion. We have revised and edited the text where we compare our approach to that of Rollins et al. (2023) and removed any comparisons regarding TCR affinity.

We acknowledge that in a natural polyclonal repertoire, each V segment is controlled by its own unique regulatory elements, and our model represents a simplification of this complexity. However, we argue that using a single endogenous promoter is a significant advance over the non-physiological, constitutive strong promoters used in prior random-integration models and represents a critical advance to recapitulate physiological TCR regulation. We have now included a discussion about how physiological the approach with inserted Vα or Vβ promoters is compared to natural gene regulation.

Dear Angelina,

Thank you again for the submission of your revised manuscript (EMBOJ-2025-121127R) to The EMBO Journal for our consideration, and for your patience during re-review. Your manuscript has now been seen by the three original referees who had previously assessed the first version of the study, and we have received their comments, which are appended below.

As you will see, referees #1 and #2 are completely satisfied with the revision, recognize that their initially raised concerns have been sufficiently addressed in a strengthened version of the manuscript, and recommend publication without any other comments. Referee #3, on the other hand, points out that certain concerns/suggestions were not addressed, but would still be supportive of the manuscript should a more complete and balanced discussion of the study's limitations be included. This referee also identifies a few additional minor issues that can all be textually addressed/clarified.

In light of this input from the referees, I would like to invite you to submit a final version of your manuscript, textually addressing the remaining points of referee #3. I would like to clarify here that no new experimental data are requested. Please also upload a point-by-point response to the remaining referee comments with detailed explanations and a description of any changes to the manuscript.

From the editorial side, there are also a few changes we need you to make in the final version of the manuscript, before we can move forward with its acceptance for publication in The EMBO Journal:

- Please remove the Figures from the main manuscript file, these should only be uploaded as individual, high-resolution, Figure files. Only the Figure legends should remain at the end of the manuscript (below the References list).

- Please change heading "Materials and Methods" to "Methods".

- Please change heading "Conflict of interest" to "Disclosure and competing interests statement".

- The author contributions statement should be removed from the manuscript file. Instead, we use CRediT to specify the contributions of each author in the journal submission system. Please feel free to use the free text box to provide more detailed descriptions during submission. See also our guide to authors for more information: https://link.springer.com/partners/embo-press/editorial-policies#Authorship.

- We kindly request you include in your resubmission a complete author checklist, which you can download from our author guidelines (https://link.springer.com/journal/44318/submission-guidelines#cms-Revised-submissions). Please note that this checklist will also be part of the Peer Review file that will be published alongside your article.

- Please note that an ORCID ID is mandatory for each co-corresponding author. No ORCID ID has been provided (in our system) for your co-author Johanne T. Jacobsen yet.

- Please update the nomenclature and callouts for your "Data Set 1" to "Dataset EV1". Please also provide a caption in a separate sheet in the same Excel file.

- We noticed that you have uploaded supporting Figures. These can either be included in the manuscript as Expanded View (EV) Figures, or in an Appendix. Expanded View Figures are collapsible/expandable online and should be cited as "Figure EV1, Figure EV2" etc. in the text, while their respective legends should be included in the manuscript file after the legends of regular ("main") Figures. Alternatively, you can bundle together all Figures (and their legends) that you do not wish to display as Expanded View Figures in a single PDF file named "Appendix", which should contain on its title page the heading "Appendix for:", followed by a Table of Contents including page numbers for all listed items. Appendix Figures should be referred to in the Appendix and throughout the main manuscript as "Appendix Figure S1", Appendix Figure S2", etc. Each Appendix Figure should be accompanied by its legend in the same PDF file. More information can be found in our guide to authors: https://link.springer.com/journal/44318/submission-guidelines#cms-Expanded-View-data.

- Please note that EMBO press papers are accompanied online by:
A) a short (2 sentences) summary of the findings and their significance,
B) 2-5 short bullet points highlighting the key results, and
C) a synopsis image in .jpg or .png format that is exactly 550 pixels wide and 300-600 pixels high (the height is variable). Please note that all text needs to be legible at the final size.
Please upload this information along with your revised manuscript (the text for A and B should be provided in a separate Word file).

- Materials and methods need to be described in the manuscript using our structured methods format, which is now required for all articles. According to this format, the Methods section includes a single "Reagents and Tools Table" -listing key reagents,

experimental models, software and relevant equipment including their sources and relevant identifiers- followed by a "Methods and Protocols" section describing the methods. Please download and fill our Reagents and Tools Table template (.docx), which you can find in our author guide: https://link.springer.com/journal/44318/submission-guidelines#cms-Revised-submissions. When submitting your revised manuscript, please do not include the Reagents and Tools Table in the Methods section of the manuscript but instead upload it as a separate file choosing the file type "Reagent Table".

- Thank you for uploading the Source Data for your Figures. We noticed that the Source Data for Figure 3M might have been mislabeled (according to your Source Data checklist, Source Data for Figure 3L have been provided instead of 3M). We would be grateful if you could please double-check and correct the labeling and/or the Source Data checklist, as appropriate.

- Please acknowledge the use of Biorender.com for creating some schematics at the end of the Methods section. Please also make sure that you have an appropriate Biorender license.

- Our data editors have checked your Figures and their legends, and raised the following queries. Please address all points below completely in your revised manuscript (all changes should be highlighted or "tracked"):
1. Please provide the exact p-values in the legends of Figures 2C, D, F, I; 3C, L, M.
2. Please note that information related to "n" is missing in the legends of Figures S2 B, S3 B, S4 B-D; S5 A, B.
3. Please note that the error bars must be defined in the legends of Figures S2 B, S3 B.

- The manuscript sections need to be named and ordered as follows: Title page - Abstract - Introduction - Results - Discussion - Methods - Data Availability - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure Legends - main Tables (if there are any) - Expanded View Figure Legends.

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Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor, The EMBO Journal
i.papaioannou@embojournal.org

Referee #1:

All reviewer queries have been adequately addressed in the revised manuscript. I congratulate the authors for this excellent study.

Referee #2:

The authors have revised the manuscript well in response to reviewers comments. The manuscript makes an important contribution to the literature.

Referee #3:

The authors chose not to address my major point on benchmarking their TCR transgenic model against retrogenic mice, which I proposed as a "compromise" in light of the fact that generating a new TCR transgenic model for a TCR for which conventional TCR transgenic models exist seemed outside the scope of a revision. While I am disappointed to see that the authors did not implement my suggestion after about nine months of revision (and apparently also did not breed mice to homozygosity in this timeframe - see response to reviewer #2), my former positive assessment also still stands that the model does provide a valuable resource for the scientific community. I could accept if the paper was to be published with the current set of data, but I would then wish for an open discussion on the limitations of the work in the discussion section. Irrespective of whether one considers the lack of consistent (i.e. with the same TCR) benchmarking against conventional TCR transgenic models a major limitation, a discussion on how TCR expression levels (and thus T cell fate after and during antigen exposure) may differ between i) conventional TCR transgenic models, ii) endogenous gene-targeted "physiological" TCR transgenic models and iii) retrogenic mice would be in my eyes highly beneficial for the discussion.

Other minor remaining points:
Fig. 1B. It is confusing that the "universal TCR targeting vector" contains the Trbv promotor while in the text only the Trav promotor is mentioned first, and in Fig. 1D and later in the text both Trav and Trbv promotors are mentioned.
Fig. 3G-H: I assume the color in the pie charts is supposed to indicate the "correct" KI TCR - this should be clearly described in the figure legend. It is interesting that also in the multimer non-binding population the frequency of the correct TCRs is relatively large. The authors speculate this could be due to competition/mispairing with the endogenous TCR, but do not actually pursue this hypothesis. They should have the single-cell sequencing data to test this concept. Also, is there anything about the non-correct TCRs that can be found that could be related to the engineering strategy or are those other TCRs seemingly completely randomly generated? Can the authors also comment on why they set the multimer gate so strictly (to the right)? I guess in cells with an intermediate multimer MFI the correctly paired TCR rate may not be 100%.

Referee #3:

The authors chose not to address my major point on benchmarking their TCR transgenic model against retrogenic mice, which I proposed as a "compromise" in light of the fact that generating a new TCR transgenic model for a TCR for which conventional TCR transgenic models exist seemed outside the scope of a revision. While I am disappointed to see that the authors did not implement my suggestion after about nine months of revision (and apparently also did not breed mice to homozygosity in this timeframe - see response to reviewer #2), my former positive assessment also still stands that the model does provide a valuable resource for the scientific community. I could accept if the paper was to be published with the current set of data, but I would then wish for an open discussion on the limitations of the work in the discussion section. Irrespective of whether one considers the lack of consistent (i.e. with the same TCR) benchmarking against conventional TCR transgenic models a major limitation, a discussion on how TCR expression levels (and thus T cell fate after and during antigen exposure) may differ between i) conventional TCR transgenic models, ii) endogenous gene-targeted "physiological" TCR transgenic models and iii) retrogenic mice would be in my eyes highly beneficial for the discussion.

We have now edited our discussion section to address these points (see new paragraph lines 359-370), and clarified the text in lines 333-334, pointing out the limitations of the TCR KI mice, in addition to the points already raised in lines 314-339.

Other minor remaining points:

Fig. 1B. It is confusing that the "universal TCR targeting vector" contains the Trbv promotor while in the text only the Trav promotor is mentioned first, and in Fig. 1D and later in the text both Trav and Trbv promotors are mentioned.

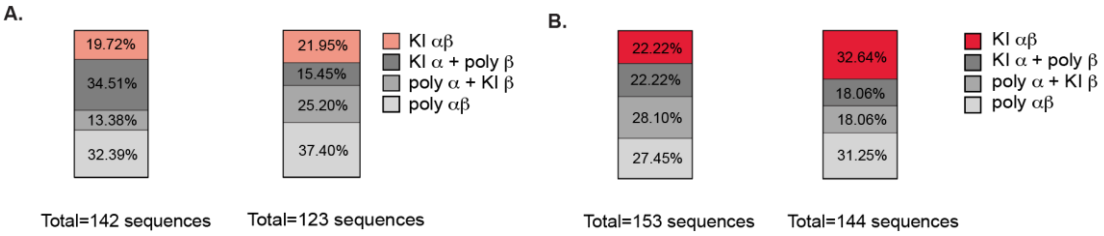
We thank the reviewer for pointing this out and have edited the figure.

Fig. 3G-H: I assume the color in the pie charts is supposed to indicate the "correct" KI TCR - this should be clearly described in the figure legend.

We agree with the reviewer that the color usage should be exlained in the figure legend. We have edited the figure legend.

It is interesting that also in the multimer non-binding population the frequency of the correct TCRs is relatively large. The authors speculate this could be due to competition/mispairing with the endogenous TCR, but do not actually pursue this hypothesis. They should have the single-cell sequencing data to test this concept. Also, is there anything about the non-correct TCRs that can be found that could be related to the engineering strategy or are those other TCRs seemingly completely randomly generated? Can the authors also comment on why they set the multimer gate so strictly (to the right)? I guess in cells with an intermediate multimer MFI the correctly paired TCR rate may not be 100%.

We analyzed our single cell TCR sequencing data to assess the pairing information among the non-binder escapees. The Reviewer Figure below shows that a large fraction of non-binders uses at least one correct chain from the knock-in but pairs with an endogenous chain, as expected. Regarding the gate, we decided to set the gates rather strictly to ensure the cells sorted properly reflected true binders and true non-binders to the best of our abilities.



Reviewer Figure. Analysis of paired TCRαβ chains among tetramer⁻ T cells from TCR KI mice. scTCR sequencing analysis of splenic T cells from TCR KI mice. A) Pairing of TCRα and β chains in non-binders of the two αKI mice sequences as shown in Fig 3G. **B**) Pairing of TCRα and β chains in non-binders of the two βKI mice sequences as shown in Fig 3H. Colored slices indicate the proportion of T cells cells using the αβ pairing of the TCR KI mice. Gray slices indicate proportion of T cells expressing either the TCRα or TCRβ or endogenous rearrangements paired with an endogenous TCR chain as indicated. Poly, polyclonal endogenous rearrangements.

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If you have any questions, please do not hesitate to contact the Editorial Office. Thank you for your contribution to The EMBO Journal. Working with you has been a pleasure.

Yours sincerely,

Ioannis Papaioannou, PhD
Editor, The EMBO Journal
i.papaioannou@embojournal.org

*****

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